THE STRUCTURE OF POLYMYXIN T_1
(STUDIES ON ANTIBIOTICS FROM THE GENUS *BACILLUS*. XXII[1])

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Amino acid analysis of the acid hydrolyzate of polymyxin T_1 revealed the amino acid composition. Isolation of the constituent amino acids and measurement of their optical activities clarified their chiralities. These were 2,4-diaminobutyric acid (6L), Thr(1L), Leu(2L) and Phe(1D). The constituent fatty acid was identified as anteisononanoic acid by gas chromatography and mass spectrometry. Deacylation with polymyxin acylase afforded deacyl polymyxin T. Successive EDMAN degradation on deacyl polymyxin T revealed most of its amino acid sequence. The chemical cleavage reaction for fragmentation of threonyl peptide on penta(DNP)-polymyxin T_1 cleaved it at the C-terminal side of the Thr residue to afford a DNP-octapeptide, whose sequence was clarified by EDMAN degradation. Thus, the structure of polymyxin T_1 was determined.

Polymyxin T_1 is a new member of polymyxin group antibiotics, produced by *Bacillus polymyxa* E-12. It is a basic substance soluble in water and is not only active against Gram-negative bacteria but also against Gram-positive bacteria. A molecular formula $C_{58}H_{102}N_{16}O_{12}\cdot 5HCl\cdot 2H_2O$ for the hydrochloride and the IR spectrum which indicates the presence of peptide bond but the absence of lactone or ester linkage have been reported[2].

Polymyxin T_1 pentahydrochloride was hydrolyzed with constant boiling hydrochloric acid. Amino acid analysis on the hydrolyzate suggested the amino acid composition of polymyxin T_1 to be Dab*(6), Thr(1), Leu(2) and Phe(1) (Table 1). 2,4-Dinitrophenylation of polymyxin T_1 gave a 2,4-dinitrophenyl derivative, which was shown to be penta(DNP)-polymyxin T_1 from releasing of γ-DNP-Dab(5), Dab(1), Thr(1), Leu(2) and Phe(1) on acid hydrolysis (Table 1). Thus, polymyxin T_1 seemed to be a decapeptide, in which five γ-amino groups of Dab residues were uncovered and an N-terminal amino group was masked. Furthermore, a ring structure with a branched chain was also suggested in which one Dab residue was present at the branching point.

When the acid hydrolyzate of polymyxin T_1 was extracted with ethyl ether, and the ethereal extract was methylated and examined by gas chromatography, a main peak of identical retention time with

Table 1. Amino acid analyses of polymyxin T_1 pentahydrochloride and penta(DNP)-polymyxin T_1

	γ-DNP-Dab	Dab	Thr	Leu	Phe
Polymyxin $T_1\cdot 5HCl$					
Found (μmoles/mg)		4.38	0.68	1.29	0.66
(moles/mole)		(6)	(1)	(2)	(1)
Penta(DNP)-polymyxin T_1					
Found (in ratio)	4.42*	1.01	0.94	2.00	1.05
(moles/mole)	(5)	(1)	(1)	(2)	(1)

* Recovery of γ-DNP-Dab by acid hydrolysis is judged to be 80~90% from other experiments.

* 2,4-Diaminobutyric acid

methyl anteisononanoate was observed as same as in polymyxin S_1[1]. The evidence was supported by gas chromatography-mass spectrometry. Thus, acyl decapeptide of a cyclic structure with a branched chain, common in polymyxin group antibiotics, was suggested.

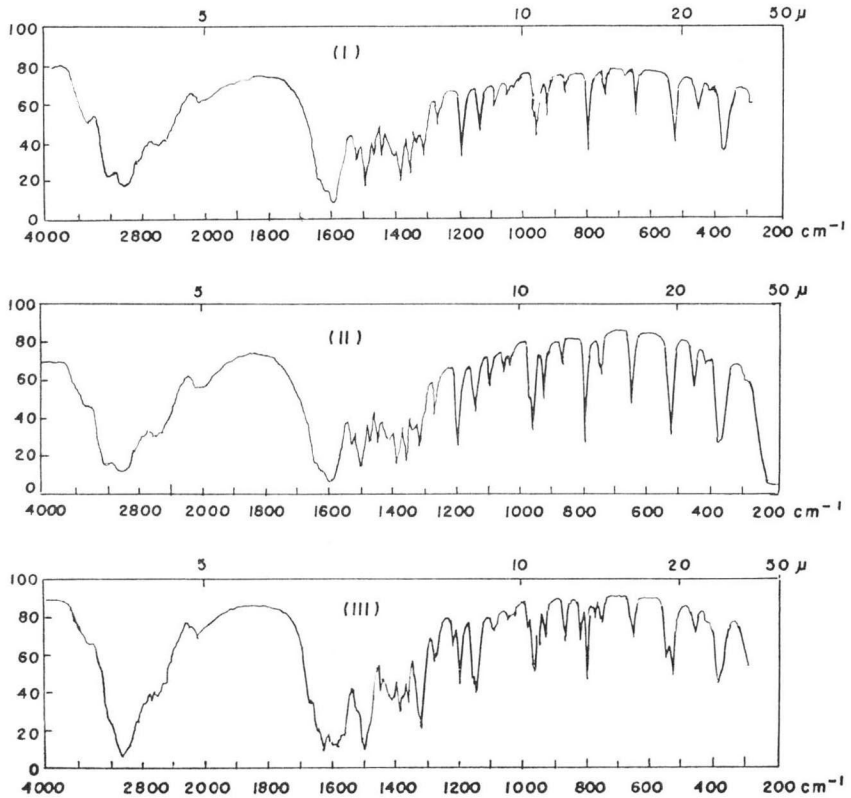
From the acid hydrolyzate of polymyxin T_1, Dab, Thr and Leu were isolated by preparative paper chromatography and Phe was isolated by a column of a porous polymer Amberlite XAD-2. Comparison of their $[M]_D$ values with those of the pure isomers and the COTTON effects in their ORD curves, indicated that all of the Dab residues as well as the Thr and the Leu residues have L-, and the Phe residue D-configurations (Table 2). The optical purity of the Dab isolated was confirmed by comparison in IR spectra with pure L-Dab and a mixture of 4L-Dab and 1D-Dab which was derived from octapeptin C_1[3]. As seen in Fig. 1, the spectrum of the isolated Dab is quite identical with that of the pure L-Dab.

Table 2. Comparison of $[M]_D$ values and COTTON effects

	$[M]_D$*	COTTON effect**
Isolated Dab	+36.5±0.9°	positive
L-Dab	+39.0±1.1°	
Isolated Thr	−12.4±4.4°	positive
L-Thr	−17.9°	
Isolated Leu	+16.1±4.5°	positive
L-Leu	+21.0°	
Isolated Phe	+14.9±4.6°	negative
L-Phe	−7.4°	

* $[M]_D$ in 5 N HCl.
** COTTON effect at *ca.* 225 nm in 0.5 N HCl.

Fig. 1. Infrared absorption spectra of 2,4-diaminobutyric acid monohydrochloride on KBr.
i) L-Dab ii) The Dab isolated from polymyxin T_1 iii) The mixture of 4L-Dab and 1D-Dab



Deacyl polymyxin T* was prepared in the same manner as in deacyl polymyxin S by enzymic cleavage with polymyxin acylase[1]. Successive EDMAN degradation on deacyl polymyxin T was carried out with the modifications described for deacyl polymyxin S[1]. However, the loss of remaining peptide was larger in this case, because this peptide contained more residues of lipophilic amino acid than deacyl polymyxin S. Therefore, the degradation did not clarify the sequence beyond the 7th step. The sequence, Dab→Thr→Dab→Dab→Dab→Phe→Leu, was indicated for the N-terminal part of the deacyl polymyxin T.

It is noteworthy for structure elucidation of this antibiotic that it contains only one Thr residue which is present at the 2nd position from the N-terminus. Several methods for selective cleavage of peptides depending on Thr residues are known. We chose the one developed by DIBELLOW *et al*[4]., because the resulting product seemed to be favorable for the EDMAN degradation. Penta(DNP)-polymyxin T_1 was oxidized with dimethylsulfoxide-dicyclohexylcarbodiimide to give a ketone, which was reacted with hydroxylamine to cause cleavage of peptide bond with the formation of an isoxazolone ring. These sequential reactions are illustrated in Fig. 2. Consequently, a DNP-octapeptide formed by cleavage at the C-terminal side of the Thr residue of penta(DNP)-polymyxin T_1 was obtained.

Fig. 2. The cleavage reaction of peptides at the threonine residue.

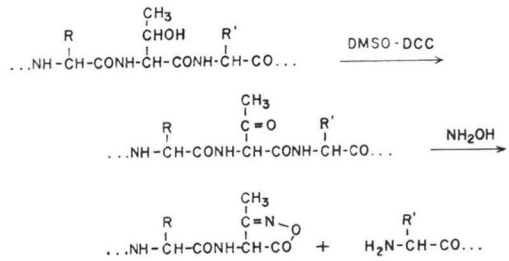


Fig. 3. The amino acid sequence of DNP-octapeptide.

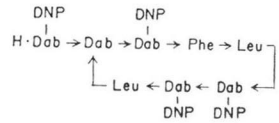


Fig. 4. The structure of polymyxin T_1.

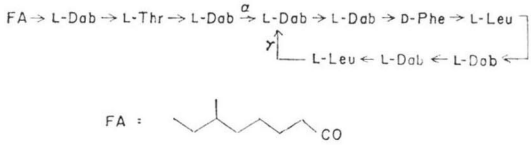
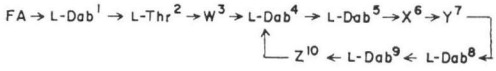


Fig. 5. The structures of polymyxin group of antibiotics.



	W	X	Y	Z
Circulin	L-Dab	D-Leu	L-Ile	L-Thr
Polymyxin E	L-Dab	D-Leu	L-Leu	L-Thr
Polymyxin M (A)	L-Dab	D-Leu	L-Thr	L-Thr
Polymyxin B	L-Dab	D-Phe	L-Leu	L-Thr
Polymyxin C or P	Dab	Phe	Thr	Thr
Polymyxin D	D-Ser	D-Leu	L-Thr	L-Thr
Polymyxin S	D-Ser	D-Phe	L-Thr	L-Thr
Polymyxin T	L-Dab	D-Phe	L-Leu	L-Leu

FA: anteisononanoyl or isooctanoyl.

EDMAN degradation on this DNP-octapeptide was carried out with the same modification in our investigation on tetra(DNP)-deacyl octapeptin C_1[3]. This reaction proceeded well, and the result indicated the sequence of this DNP-octapeptide as shown in Fig. 3. Furthermore, when the remaining peptide of the 1st step of the EDMAN degradation, a cyclic heptapeptide, was dinitrophenylated and then hydrolyzed, approximately one mole of α-DNP-Dab was found in addition to the other residues. This provided conclusive

* The subscript number, which implied difference of the fatty acyl residue, is not needed for the deacyl derivative.

evidence for the mode of branching in the peptide ring. In accidentally, the structure of this DNP-octapeptide (Fig. 3) is identical with that of tetra(DNP)-octapeptin C_1 except for the chirality of one Dab residue.

By superimposing the above two sequences, we could draw the total structure of polymyxin T_1 as in Fig. 4.

Many efforts were devoted to elucidation of the structures of polymyxin group of antibiotics, particulary by SUZUKI and his co-workers[5]. Most of these have been reviewed by VOGLER and STUDER in 1966[6]. Recently, the structure of polymyxin M has been established.[7] The identity of polymyxins A and M were considered as established by WILKINSON and LOWS[8] from their amino acid and fatty acid composition, except for the configuration of one Dab residue[8]. So far polymyxins C and P have not been subjected to structural studies. However, recently we have isolated an antibiotic which is identical with polymyxin C or P with respect to amino acid composition, and we determined its amino acid sequence (unpublished data). As reported in preceding paper[1] and here, we could add two new members of this group of antibiotics. Thus, now all structurally defined members of the polymyxin group antibiotics can be drawn as in Fig. 5 with respect to their peptide parts. It has been mentioned earlier[6], that there is the general type of structures for polymyxin group of antibiotics, in which each member differs in the structure of fatty acid and the variations in the amino acid residues at the 3, 6 and 7th position, W, X and Y in Fig. 5. Now, we could show, in polymyxin T_1, a new variation at the 10th position, Z in Fig. 5. It should be noticed that polymyxin T_1 is unique in this respect and it exhibits somewhat different antimicrobial properties from other members. Furthermore, it is of interest to note that the structure of the cyclic heptapeptide part of polymyxin T_1 is closely similar to those of octapeptin antibiotics which also have been isolated in recent years[9]. Very recently, another new member of polymyxin group antibiotics, polymyxin F, has been isolated by PARKER *et al*,[10] whose amino acid composition is Dab(5), Ser(1), Ile(1), Leu(2) and Thr(1).

Experimental

The experimental procedures including amino acid analysis are the same as those described in the preceding paper.[1]

Amino acid composition of polymyxin T_1

A few mg of polymyxin T_1 pentahydrochloride was hydrolyzed and the hydrolyzate was analyzed with an automatic amino acid analyzer in the same manner as in polymyxin S_1. The amino acids found are presented in Table 1.

Penta(DNP)-polymyxin T_1

Polymyxin T_1 pentahydrochloride (30 mg) and $NaHCO_3$ (50 mg) were dissolved in water (0.3 ml). To the solution 0.6 ml of 10% 2,4-dinitrofluorobenzene ethanol solution was added, and the mixture was stirred for 16 hours in the dark. Saturated $NaHCO_3$ solution was added to the reaction mixture, which was then extracted with ethyl acetate. The ethyl acetate extract was washed with dil. $NaHCO_3$ solution, water, dil.HCl solution and saturated NaCl solution, and then dried (Na_2SO_4). The residue obtained by evaporation of the solvent was repeatedly triturated with ether, resulting in a yellow powder (41 mg) of penta(DNP)-polymyxin T_1. The amino acids found with the acid hydrolyzate are in Table 1.

The constituent fatty acid

The ethereal extract of the acid hydrolyzate of polymyxin T_1 was methylated and analyzed with gas chromatography in the same manner as in polymyxin S_1, and the same result was obtained. A main peak of identical retention time with methyl anteisononanoate was given, and the identity was con-

firmed with GC-MS. The ion peaks contained in the GC-MS were the same as described in the preceding paper[1].

Isolation of constituent amino acids

Polymyxin T_1 pentahydrochloride (146 mg) was hydrolyzed with constant boiling hydrochloric acid at 110°C for 20 hours. The hydrolyzate was extracted with ethyl ether, the extract was used for the analysis of fatty acid and the aqueous solution was concentrated to dryness. The residue, the amino acid mixture, was applied to two sheets of Toyo Roshi No. 51, 60×60 cm, and developed with *n*-butanol - acetic acid - water (4: 1: 2). Three ninhydrin-positive zones (Rf: *ca.* 0.08, 0.26 and 0.61) separated were extracted with water and followed as below.

The fraction of Rf 0.08 containing Dab was adsorbed on a small column of Dowex 50×8 (NH_4) at acid pH, the column was washed with water and eluted with 0.3 N NH_4OH. The residue by evaporation of the eluate was dissolved in dil.HCl and then freeze-dried. The residue was crystallized from water and ethanol, to give colorless crystals (61 mg) of 2,4-diaminobutyric acid monohydrochloride.

Anal. Found: C, 31.31; H, 7.42; N, 17.86; Cl, 23.04.
Calcd. for $C_4H_{10}N_2O_2 \cdot HCl$: C, 31.07; H, 7.17; N, 18.12; Cl, 22.93.

$[\alpha]_D^{22.5}$ +23.6±0.6° (*c* 1.020, 5 N HCl). ORD: $[\phi]_{250}$ +610, $[\phi]_{225}$ +1750; $[\phi]_{211.5}$ 0. CD: $[\theta]_{245}$ 0, $[\theta]_{210}$ +2970: $[\theta]_{203}$ +2510 (*c* 0.2070, 0.5 N HCl).

The fraction of Rf 0.26 containing Thr was adsorbed on a Dowex 50×8 (NH_4) column and eluted with 0.3 N NH_4OH as above. The residue obtained by evaporation of the eluate was crystallized from water and ethanol, to yield colorless crystals (7.1 mg) of threonine.

Anal. Found: C, 40.04; H, 7.89; N, 11.65.
Calcd. for $C_4H_9NO_3$: C, 40.33; H, 7.62; N, 11.76.

$[\alpha]_D^{22.5}$ −10.3 ±3.7° (*c* 0.136, 5 N HCl). ORD: $[\phi]_{250}$ +380, $[\phi]_{227}$ +1900, $[\phi]_{214.5}$ 0. CD: $[\theta]_{245}$ 0, $[\theta]_{210}$ +5010, $[\theta]_{203}$ +4150 (*c* 0.1630, 0.5 N HCl).

The fraction of Rf 0.61 containing Leu and Phe was dissolved in 2 ml of water, and passed through an Amberlite XAD-2 column (1.6×28 cm). Upon washing with water Leu appeared in the fraction of 42~90 ml and Phe in 128~218 ml. The fractions were processed by a Dowex 50×8 (NH_4) column as above, and leucine (15.4 mg) and phenylalanine (9.5 mg) were obtained as colorless crystals.

Leucine:

Anal. Found: C, 54.88, H, 10.18; N, 10.55.
Calcd. for $C_6H_{13}NO_2$: C, 54.93; H, 9.99; N, 10.68.

$[\alpha]_D^{22.5}$ +12.3 ±3.4° (*c* 0.154, 5 N HCl). ORD: $[\phi]_{250}$ +910, $[\phi]_{225}$ +3160, $[\phi]_{212}$ 0. CD: $[\theta]_{245}$ 0, $[\theta]_{210.5}$ +5370, $[\theta]_{202}$ +3430 (*c* 0.2745, 0.5 N HCl).

Phenylalanine:

Anal. Found: C, 65.70; H, 6.68; N, 8.46.
Calcd. for $C_9H_{11}NO_2$: C, 65.43; H, 6.71; N, 8.48.

Table 3. EDMAN degradation reaction of deacyl polymyxin T

	PTH-Amino acid	Amino acid found (in ratio)			
		Dab	Thr	Leu	Phe
Original peptide	—	5.93	0.97	2.00	0.87
1st step	γ-PTC-Dab*	4.57	0.72	2.00	0.96
2nd step	Thr, ⊿Thr	4.54	0.16	2.00	1.03
3rd step	γ-PTC-Dab	3.89	0.13	2.00	0.99
4th step	—	3.08	0.0	2.00	0.94
5th step	γ-PTC-Dab	1.81	0.0	2.00	0.88
6th step	Phe	1.81	0.0	2.00	0.56
7th step	Leu	—	—	—	—

* Phenylthiohydantoin of γ-phenylthiocarbamyl-2,4-diaminobutyric acid.

$[\alpha]_D^{22.0}$ +9.0 ±2.8° (*c* 0.1780, 5 N HCl). ORD: $[\phi]_{250}$ −960, $[\phi]_{225}$ −4930, $[\phi]_{220}$ −2970. CD: $[\theta]_{245}$ 0, $[\theta]_{219}$ −12700, $[\theta]_{205}$ −3370 (*c* 0.2959, 0.5 N HCl).

Deacyl polymyxin T

Polymyxin T_1 pentahydrochloride (35 mg) was dissolved in 0.05 M phosphate buffer, pH 7.2. Polymyxin acylase (15 mg) was added to the solution, which was stirred for 20 hours at 37°C. The reaction mixture was acidified to pH 2.0 (HCl) and centrifuged. The supernatant was subjected to paper chromatography on Toyo Roshi No. 51, *n*-butanol - acetic acid - water (4: 1: 2). A ninhydrin - positive zone with Rf *ca.* 0.22 was extracted with water. Lyophilization of the extract gave a colorless powder (26 mg) of deacyl polymyxin T. Amino acids found on the acid hydrolyzate were (μmoles/mg): Thr (0.50), Leu (0.90), Phe (0.47) and Dab (3.04).

Table 4. EDMAN degradation of DNP-octapeptide

	PTH-AA	Amino acid found (in ratio)			
		Dab	γ-DNP-Dab	Leu	Phe
Orig.	—	1.02	3.69	2.00	1.10
1st	γ-DNP-Dab	1.00	2.58	2.00	1.06
2nd	—	0.02	2.57	2.00	1.06
3rd	γ-DNP-Dab	0.12	1.75	2.00	1.01
4th	Phe	0.13	1.88	2.00	0.23
5th	Leu	0.07	1.53	1.00	0.11
6th	γ-DNP-Dab	0.08	0.97	1.00	0.14
7th	γ-DNP-Dab	0.11	0.69	1.00	0.25
8th	Leu	—	—	—	—

EDMAN degradation of deacyl polymyxin T

Successive EDMAN degradations on deacyl polymyxin T were carried out in the same manner as that in deacyl polymyxin S described in the preceding paper.[1] About 6 μmole of deacyl polymyxin T was subjected to the reaction, and the following result was obtained (Table 3). The quantity of the remaining peptide after the 7th step of this reaction became so small, that no significant result was obtained hereafter. From this, the sequence, Dab→Thr→Dab→Dab→Dab→Phe→Leu, was indicated for the N-terminal part of deacyl polymyxin T.

Cleavage of penta(DNP)-polymyxin T_1

Penta(DNP)-polymyxin T_1 (22 mg) was dissolved in dimethyl sulfoxide (0.25 ml), trifluoroacetic acid (2.5 μl) and pyridine (5 μl), and dicyclohexylcarbodiimide (DCC) (44 mg) was added. After stirring for a short time, the mixture was allowed to stand for 16 hours at room temperature. The reaction mixture was diluted with ethyl acetate. The excess DCC was decomposed with oxalic acid (44 mg), and the resulting precipitate was filtered off. The ethyl acetate solution was washed with saturated $NaHCO_3$ solution and then saturated NaCl solution, and dried (Na_2SO_4). The residue obtained by evaporation of ethyl acetate was triturated with benzene, giving a yellow powder of an intermediate ketone derivative. The yellow powder was then dissolved in acetic acid (1.35 ml), methanol (0.54 ml) and water (0.27 ml). After addition of hydroxylamine hydrochloride (20 mg), the solution was heated at 80°C for 40 minutes. The reaction mixture was diluted with ethyl acetate, washed with saturated $NaHCO_3$ solution, water, 1 N HCl and saturated NaCl solution, and dried (Na_2SO_4). After concentration to dryness, the residue was subjected to TLC on silica gel with chloroform - methanol (4: 1). The reaction product to be pursued remained at the origin, while unreacted ones and the probable N-terminal fragment migrated further up, the latter showing an Rf *ca.* 0.47. The yellow zone at the origin was extracted with a mixture of chloroform and methanol (1: 1), the extract was evaporated and then dissolved in ethyl acetate. The ethyl acetate solution was washed with 1 N HCl and saturated NaCl solution, dried (Na_2SO_4), and concentrated to dryness. Trituration of the residue with ethyl ether gave a yellow powder (4.0 mg). It was judged to be the DNP-octapeptide formed by cleavage at the C-terminal side of the Thr residue of penta(DNP)-polymyxin T_1, because the acid hydrolyzate showed the following amino acids (in ratio): Dab(1.02), γ-DNP-Dab(3.69), Leu(2.00) and Phe(1.10).

EDMAN degradation of the DNP-octapeptide

Successive EDMAN degradation on the DNP-octapeptide obtained as above was carried out with the same modification as in our investigation on tetra(DNP)-deacyl octapeptin C_1[3]. The modification was as follows: excess PTC was removed by extracting with cyclohexane twice and then benzene twice, PTC-amino acid was extracted with ethyl ether, and the PTC-peptide in the 2nd step was heated

in TFA at 40°C for 180 minutes and further heated at 80°C for 10 minutes in a mixture of acetonitrile - 2 N HCl (1: 1). The product was used for the next step without purification.

Approximately 1.8 μmole of the DNP-octapeptide was subjected to the reaction, and the result shown in Table 4 was obtained. Furthermore, a portion (0.15 μmole) of the remaining peptide at the 1st step of the reaction was examined as follows: It was dinitrophenylated in a mixture of water (30 μl), ethanol (120 μl), ethyl acetate (100 μl), $NaHCO_3$ (2.4 mg) and 10% 2,4-dinitrofluorobenzene ethanol solution (20 μl) at room temperature for 16 hours. The reaction mixture was then diluted with saturated $NaHCO_3$ solution and extracted with ethyl acetate. The ethyl acetate solution was washed with saturated $NaHCO_3$ solution, water, 1 N HCl and saturated NaCl solution successively, and dried (Na_2SO_4). The residue obtained by evaporation of the solvent was repeatedly washed with petroleum ether to remove unreacted 2,4-dinitrofluorobenzene. The dinitrophenylated product thus obtained showed a single spot with Rf 0.52 on a silica gel plate with chloroform - methanol (9: 1), and by acid hydrolysis released the following amino acids (in ratio): α-DNP-Dab (0.66), γ-DNP-Dab (2.47), Leu(2.00) and Phe(1.05).

These results clearly indicated the sequence of the DNP-octapeptide as shown in Fig. 3.

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References

1) SHOJI, J.: T. KATO & H. HINOO: The structure of polymyxin S_1. (Studies on antibiotics from the genus *Bacillus*. XXI). J. Antibiotics 30; 1035~1041, 1977
2) SHOJI, J.; H. HINOO, Y. WAKISAKA, K. KOIZUMI, M. MAYAMA & S. MATSUURA: Isolation of two new polymyxin group antibiotics. (Studies on antibiotics from the genus *Bacillus*. XX). J. Antibiotics 30: 1029~1034, 1977
3) KATO, T. & J. SHOJI: The amino acid sequence of octapeptin C_1 (333-25). (Studies on antibiotics from the genus *Bacillus*. XIX). J. Antibiotics 29: 1339~1340, 1976
4) DIBELLO, C.; F. FILIRA, V. GIORMANI & F. D'ANGELI: β-Carbonylamides in peptide chemistry. Further studies on the oxidation and cleavage of threonyl dipeptide. Gazz. Chim. Ital. 102: 180~188, 1972
5) HAYASHI, K.; Y. SUKETA & T. SUZUKI: Chemical structure of circulin B. Experientia 24: 656~657, 1968
6) VOGLER, K. & R. O. STUDER: The chemistry of the polymyxin antibiotics. Experientia 22: 345~354, 1966
7) SILAEV A. B.; S. N. MAEVSKAYA, ZH. P. TRIFONOVA, E. P. YULIKOVA, N. M. VASILÉVA & G. S. KATRUKHA: Chemistry of polymyxin M. XIX. Structure of polymyxin M. J. General Chemistry USSR 45: 2287~2291, 1975
8) WILKINSON, S. & L. A. LOWS: Structures of polymyxins A and the question of identity with the polymyxin M. Nature 212: 311, 1966
9) MEYERS, E.; W. L. PARKER, W. E. BROWN, J. SHOJI & Y. WAKISAKA: A nomenclature proposal for the octapeptin antibiotics. J. Antibiotics 29: 1241~1242, 1976
10) PARKER, W. L.; M. L. RATHNUM, L. D. DEAN, M. W. NIMECK, W. E. BROWN & E. MEYERS: Polymyxin F, a new antibiotic. J. Antibiotics 30: 767~769, 1977